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<p>(21) International Application Number: PCT/US99/26004 (22) International Filing Date: 17 November 1999 (17.11.99) (30) Priority Data: 09/195,367 18 November 1998 (18.11.98) US (71) Applicant: CANJIL, INC. [US/US]; 3525 John Hopkins Court, San Diego, CA 92121 (US). (72) Inventor: WILLIS, Kenneth, N.; 821 Bluffcrest Lane, Encinitas, CA 92024 (US). (74) Agents: MURPHY, Richard, B. et al.; Schering-Plough Corporation, Patent Department, K-6-1, 1990, 2000 Galloping Hill Road, Kenilworth, NJ 07033-0530 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LU, LV, MA, MD, MG, MK, MN, MX, NO, NZ, PL, PT, RO, RU, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UZ, VN, YU, ZA. ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: VIRAL VECTORS WITH LATE TRANSGENE EXPRESSION (57) Abstract The present invention is directed to recombinant viruses containing a therapeutic transgene operably linked to a late regulatory element. The vectors of the present invention are capable of replication and lysis of neoplastic cells. The vectors may optionally include modifications to the genome so as to impart additional therapeutic, conditionally replicating or targeting functions. The present invention also provides pharmaceutical formulations of such vectors. The present invention further provides methods of use of such vectors. The present invention further provides methods of preparing the vectors.</p>		

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TITLE

VIRAL VECTORS WITH LATE TRANSGENE EXPRESSION

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BACKGROUND OF THE INVENTION

The genome of the adenovirus has been well characterized. This information has been used to design recombinant adenoviruses capable of acting as vectors for the introduction of exogenous DNA into target cells. Many of these viral vectors contain modifications to the early gene products to endow the vectors with specific activities.

- 10 For example, the early genes E1 and E2, are transcribed early after infection. The products of these genes are responsible for the suppressing the ability of the infected cell to respond to the infection and to promote viral replication in the host.

- In particular, the immediate early gene E1a is transcribed rapidly following infection. The E1a product can immortalize primary cells *in vivo*. E1a has also been
15 show to bind a cellular protein p105 RB, the product of the retinoblastoma gene. The E1b gene is not capable of transforming cells on its own, but cooperates with E1a to stably transform cells. Both E1a and E1b are necessary for the full transformation and tumor formation in animals. The E1a gene has a variety of functions. The full scope of activity of the adenovirus E1a region is described in Bayley, S. and Mymryk, J. (1994)
20 Intl. J. of Oncology 5:425-444.

- The E1b genes are known to interact with host cell proteins. In particular two proteins are produced by differential splicing of the E1b sequence, p19 and p55. The p55 protein has been well characterized as interacting with the p53 gene product. p53 is a well characterized protein and has been shown to activate programmed cell death
25 (PCD) apoptotic pathways when present in sufficient intracellular concentration. p53 induced apoptosis has been shown to take place in response to a wide variety of cell injury including radiation, DNA damaging agents, etc. By binding to p53, p55 prevents the formation of the active p53 phosphorylated tetramer. Since p53 is sequestered by p55, a primary apoptotic pathway is never initiated and the infected cell
30 undergoes uncontrolled replication in combination with Rb activity in response to the E1a 12S and 13S proteins.

- E1a and E1b cooperate in their activities. In particular E1a has been shown to induce p53 dependent and p53 independent apoptosis. In addition to inducing other early viral genes, E1a drives quiescent cell into S-phase which is required for viral replication. It is believed that this early pressure to enter S-phase by the virus on the quiescent cell induces a apoptotic response from the cell. The adenovirus responds by the expression of the E1b 55K protein to bind to p53 and delay the induction of apoptosis so that the virus is able to replicate prior to the death of its host. Adenovirus also produced the E1B19K protein which prevents or delays E1a mediated apoptosis as well.
- Alternative to this type of selectively replicating vector is the employment of a replication deficient adenoviral vector containing extensive elimination of E1 function. In particular, vectors containing elimination of E1, E3 and partial E4 deletions have been employed to deliver exogenous transgenes. Such vectors have been employed to deliver the p53 gene to target cells. It has been demonstrated that the expression of an exogenously administered wild type p53 in a p53 deficient (p53 mutated or p53 null) tumor cell is capable of inducing p53 mediated apoptosis in the tumor cell. Such viral vectors for the delivery of p53 are currently under development Schering Corporation. Again these vectors have demonstrated acceptable toxicology profiles and therapeutic efficacy for human therapeutic applications and are in Phase II clinical trials in man for the treatment of p53 related malignancies.

- Replication deficient and selectively replicating vectors have, at least in theory, design drawbacks which are of concern to clinicians. Because the replication deficient vectors will not propagate uncontrollably in the patient, they have a more theoretically appealing safety profile. However, as effective tumor elimination requires the infection of the substantial majority of the tumor cells being infected, a substantial molar excess of vector is commonly used to insure therapeutic effectiveness. Selectively replicating vectors are viewed as being more of an issue from a safety perspective because of their ability to replicate and potentially mutate to form fully replication competent vectors in the patient. However, by exploiting the natural ability to the virus to propagate under particular conditions enables these vectors to spread to surrounding tumor cells. Since the vectors themselves are able to replicate, a lower initial dose of such vectors is

required. This is favorable from an immunological perspective as well as for economic reasons in the manufacture of such agents.

In order to facilitate the understanding of the present invention, a brief overview of the life cycle of a typical virus used for delivery of exogenous transgenes, the
5 adenovirus, is offered. The adenoviral replicative cycle in human cells can be divided into the early and late phase which are punctuated by the onset of viral DNA replication. The early phase begins when viral particles attach to cells through interaction between the virion fiber domain and cell surface receptors. The virion moves into the cell by
10 either endocytosis or direct penetration of the cytoplasmic membrane and is transported to the nucleus where most of the capsid is shed. In the nucleus, the virion core proteins are removed yielding viral chromosomes that are almost entirely devoid of virion proteins. Expression of the viral genome is temporally coordinated and begins with the E1a region about one hour after infection. The other early genes E1b, E2, E3 and E4 are first expressed soon after E1a at the 1.5-2.0 hours post infection. A number of the
15 protein products encoded by the early genes are required for viral DNA replication, while others prepare the DNA synthesis machinery of the infected cell for efficient viral DNA replication. Some early virally encoded proteins have been associated with protecting infected cells from immune surveillance.

The late phase of infection with onset of DNA replication at about 7 hour post
20 infection. In the native adenovirus, the messenger RNAs for all late gene products are spliced from a primary RNA which is transcribed from the major late promoter (MLP). The MLP is located at position 16.5 on the r-strand. Although the major late promoter is active to a limited extent in the early phase of infection, the transcription does not proceed past map position 39. During the late phase of the viral life cycle, the MLP is
25 fully activated and continues to map position 99. Each late primary RNA transcript is processed into one of five different mRNAs, L1-L5. These mRNAs all contain a common tripartite leader sequence of 203 nucleotides. Late mRNAs encode capsid components and proteins required for assembly of virions and packaging of the viral chromosome. Viral DNA replication requires the terminal protein for initiation and
30 proceed by a semi-conservative mechanism. With the onset of replication, efficient transcription of the late gene families from the major late promoter begins and attains a maximal level approximately 18 hours post infection. During the late phase viral

- proteins block cellular DNA and protein synthesis, presumably so that maximum viral macromolecular synthesis can occur. Intermediate gene expression, which actually begins during the early phase, reaches a maximum between 8 - 12 hour post infection. Assembly of the virion and packaging of the viral genome begins at about 24 hours after infection. Infected cells are killed because of attrition and lyse yielding approximately 10,000 virions per cell.

SUMMARY OF THE INVENTION

- The present invention provides a conditionally replicating recombinant virus containing a therapeutic transgene under control of a late regulatory element. The invention further provides pharmaceutical formulations and methods of use of same. The present invention further provides recombinant producer cells capable of complementing the packaging genes defective or deleted in the vector. The present invention also provides method of making such vectors and formulations.

BRIEF DESCRIPTION OF THE FIGURES

- Figure 1 is an autoradiogram of a time course study comparing the levels of p53 expression of a replication competent MLP-p53 (Ad5dE1bMLPp53) virus in comparison to the replication deficient p53 vector (ACN53) at two different concentrations (1.8×10^8 particles/ml (upper panel) and 1.8×10^9 particles/ml (lower panel) in MRC9 cells according to the procedures of Example 2.a. herein. As can be seen from the data presented, the replication competent MLP-p53 construct resulted in expression later in time than the replication deficient CMV-p53 virus (ACN53). The data further illustrates that the levels of p53 expression produced from the replication competent MLP-p53 virus are significantly greater than the replication deficient CMV-p53 virus.
- Figure 2 presents the results of an experiment substantially similar to that presented in Figure 1 except that the experiment was conducted in SK-HEP1 hepatocellular carcinoma cells according to the procedures of Example 2.b. herein. Again, the time course experiment demonstrates the temporal and greater expression of p53 in the replication competent MLP-p53 construct.
- Figure 3 presents the results of an experiment substantially identical to that presented in Figure 1 except that the experiment was conducted in NCI H358 breast

cancer cells according to the procedures of Example 2.c. herein. Again, the time course experiment demonstrates the temporal expression of p53 in the replication competent MLP-p53 construct.

Figure 4 presents the results of an experiment similar to that presented in Figure 3 except that a replication competent E1B Δ 55K-CMV-p53 virus was included for comparison and only a single dose was administered according to the procedures of Example 2.d. herein. This time course experiment demonstrates the temporal expression of p53 in the replication competent MLP-p53 construct as compared to the substantially similar construct wherein the p53 gene was under control of the constitutive CMV promoter. This data confirms that the MLP-p53 construct is indeed expressing the p53 in a temporal manner late in the viral replication cycle.

Figure 5 is a digest of viral DNA from SK-BR3 cells infected with a one hour pulse of the indicated viruses at a concentration of 1.8×10^9 particles/ml and harvested approximately 48 hours later according to the procedures of Example 2.d. herein. The results presented demonstrate that replication competent wild-type Ad5 (Ad5WT), replication competent E1B Δ 55K (ZAZA) virus and replication competent E1B Δ 55K-MLP-p53 (55K/MLP53) virus all replicate their viral DNA well while the replication deficient adenovirus control (rAdcon) and the replication deficient vector encoding p53 (FTCB) does not.

Figure 6 is a graphical representation of the data obtained *in vivo* in a PC-3 mouse tumor model. Tumor volume is plotted on the vertical axis and days following administration is plotted on the horizontal axis. As can be seen from the data presented, the replicating virus E1B Δ 55K-MLP-p53 (cFAMA) was able to produce tumor regression in an *in vivo* mouse model of human cancer. The replication competent CMV driven p53 virus also replicates its viral DNA, but to a lesser extent.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element.

1. Replication Competent Recombinant Virus:

The term "replication competent" is made in reference to a virus which is capable of replicating its genome and packaging the replicated viral genome into

infectious particles in mammalian cells. It should be noted that the term replication competent does not generally apply to virus that can only be grown in cells which have been modified to provide deleted viral functions in trans.

- The term "recombinant virus" refers to any of the obligate intracellular parasites
- 5 having no protein-synthesizing or energy-generating mechanism capable of infecting a mammalian cell whose genomes have been modified by conventional recombinant DNA techniques. The viral genome may be RNA or DNA contained within a coated structure of protein or a lipid membrane. The terms virus(es) and viral vector(s) are used interchangeably herein. The viruses useful in the practice of the present invention
- 10 include recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridae, parvoviridae, picornaviridae, herpesviridae, poxviridae, adenoviridae, or picornaviridae. Chimeric viral vectors which exploit advantageous elements of each of the parent vector properties (See e.g., Feng, *et al.* (1997) *Nature Biotechnology* 15:866-870) may also be useful in the practice of the
- 15 present invention. Minimal vector systems in which the viral backbone contains only the sequences need for packaging of the viral vector and may optionally include a transgene expression cassette may also be produced according to the practice of the present invention. Although it is generally favored to employ a virus from the species to be treated, in some instances it may be advantageous to use vectors derived from
- 20 different species which possess favorable pathogenic features. For example, equine herpes virus vectors for human gene therapy are described in WO98/27216 published August 5, 1998. The vectors are described as useful for the treatment of humans as the equine virus is not pathogenic to humans. Similarly, ovine adenoviral vectors may be used in human gene therapy as they are claimed to avoid the antibodies against the
- 25 human adenoviral vectors. Such vectors are described in WO 97/06826 published April 10, 1997.

- In the preferred practice of the invention, the virus is an adenovirus. The term "adenovirus" is synonymous with the term "adenoviral vector" and refers to viruses of the genus adenoviridae. The term adenoviridae refers collectively to animal
- 30 adenoviruses of the genus mastadenovirus including but not limited to human, bovine, ovine, equine, canine, porcine, murine and simian adenovirus subgenera. In particular, human adenoviruses includes the A-F subgenera as well as the individual serotypes

thereof the individual serotypes and A-F subgenera including but not limited to human adenovirus types 1, 2, 3, 4, 4a, 5, 6, 7, 8, 9, 10, 11 (Ad11A and Ad 11P), 12, 13, 14, 15, 16, 17, 18, 19, 19a, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 91. The term bovine adenoviruses includes but is not limited to bovine adenovirus types 1, 2, 3, 4, 7, and 10. The term canine adenoviruses includes but is not limited to canine types 1 (strains CLL, Glaxo, RI261, Utrecht, Toronto 26-61) and 2. The term equine adenoviruses includes but is not limited to equine types 1 and 2. The term porcine adenoviruses includes but is not limited to porcine types 3 and 4. The term recombinant adenovirus also includes chimeric (or even multimeric) vectors, i.e. vectors constructed using complementary coding sequences from more than one viral subtype. See, e.g. Feng, *et al.* Nature Biotechnology 15:866-870.

In the preferred practice of the invention, the recombinant adenoviral vector is derived from genus adenoviridae. Particularly preferred viruses are derived from the human adenovirus type 2 or type 5. In the preferred practice of the invention as exemplified herein, the preferred vector is derived from the human adenoviridae. More preferred are vectors derived from human adenovirus subgroup C. Most preferred are adenoviral vectors derived from the human adenovirus serotypes 2 and 5. In the most preferred practice of the invention the virus is derived human adenovirus Type 5 dj309, dj327, dj520 or wild-type adenovirus.

II. Late Regulatory Element

The term "late regulatory element" refers to regulatory element which drives transcription of the therapeutic transgene a point later in time than the element which induces initial viral replication. Characteristically, these promoter elements are found driving expression of packaging proteins and other proteins late in the viral life cycle. An example of such late regulatory element when the parent vector is adenovirus is the adenovirus major late promoter (MLP). Other viral vector systems also possess late temporally regulated promoters. For baculoviral vectors, the AcNPV basic gene promoter and the polyhedrin gene promoters may be employed (Sridhar, *et al.* (1993) FEBS Lett. 315:282-286. For herpes simplex viruses, the Latent Activated Promoters may be employed. See, e.g. Rivera-Gonzalez, *et al.* (1994) Virology 202:550-564 and Imbal, *et al.* (1992) J. Virol. 66:5453-5463. For human papilloma viruses, the

- HPV31b promoter may be employed (Ozbun and Meyers (1998) J. Virol. 72:2715-22). For parvoviruses, the P39 promoter. For vaccinia virus, the p11 promoter. In the preferred practice of the invention, the virus is derived from the genus adenoviridae and the late regulatory element is the adenoviral Major Late Promoter. The Major Late Promoter is well characterized in the art and resides at approximately map position 16.5 of the adenoviral Type 2 genome.

III. Operably Linked:

- The term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the nucleotide sequences being linked are typically contiguous. However, as enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not directly flanked and may even function in trans from a different allele or chromosome.

IV. Therapeutic Transgene:

- The term "therapeutic transgene" refers to a nucleotide sequence the expression of which in the target cell produces a therapeutic effect. The term therapeutic transgene includes but is not limited to tumor suppressor genes, antigenic genes, cytotoxic genes, cytostatic genes, pro-drug activating genes, apoptotic genes, pharmaceutical genes or anti-angiogenic genes. The vectors of the present invention may be used to produce one or more therapeutic transgenes, either in tandem through the use of IRES elements or through independently regulated promoters.

A. Tumor Suppressor Gene

- The term "tumor suppressor gene" refers to a nucleotide sequence, the expression of which in the target cell is capable of suppressing the neoplastic phenotype and/or inducing apoptosis. Examples of tumor suppressor genes useful in the practice of the present invention include the p53 gene, the APC gene, the DPC-4 gene, the BRCA-1 gene, the BRCA-2 gene, the WT-1 gene, the retinoblastoma gene (Lee, *et al.*

- (1987) Nature 329:642), the MMAC-1 gene, the adenomatous polyposis coli protein (Albertsen, *et al.*, United States Patent 5,783,666 issued July 21, 1998), the deleted in colon carcinoma (DCC) gene, the MMS-2 gene, the NF-1 gene, nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. (Cheng, *et al.* 5 1998. Proc. Nat. Acad. Sci. 95:3042-3047), the MTS1 gene, the CDK4 gene, the NF-1 gene, the NF2 gene, and the VHL gene.

B. Antigenic Genes

- The term "antigenic genes" refers to a nucleotide sequence, the expression of which in the target cells results in the production of a cell surface antigenic protein 10 capable of recognition by the immune system. Examples of antigenic genes include carcinoembryonic antigen (CEA), p53 (as described in Levine, A. PCT International Publication No. WO94/02167 published February 3, 1994). In order to facilitate immune recognition, the antigenic gene may be fused to the MHC class I antigen.

C. Cytotoxic Genes

- 15 The term "cytotoxic gene" refers to nucleotide sequence, the expression of which in a cell produces a toxic effect. Examples of such cytotoxic genes include nucleotide sequences encoding pseudomonas exotoxin, ricin toxin, diphtheria toxin, and the like.

D. Cytostatic Genes

- 20 The term "cytostatic gene" refers to nucleotide sequence, the expression of which in a cell produces an arrest in the cell cycle. Examples of such cytostatic genes include p21, the retinoblastoma gene, the E2F-Rb gene, genes encoding cyclin dependent kinase inhibitors such as P16, p15, p18 and p19, the growth arrest specific homeobox (GAX) gene as described in Branellec, *et al.* (PCT Publication 25 WO97/16459 published May 9, 1997 and PCT Publication WO96/30385 published October 3, 1996).

E. Cytokine Gene

- The term "cytokine gene" refers to a nucleotide sequence, the expression of which in a cell produces a cytokine. Examples of such cytokines include GM-CSF, 30 interleukins, especially IL-1, IL-2, IL-4, IL-12, IL-10, IL-19, IL-20, interferons of the

alpha, beta and gamma subtypes especially interferon α -2b and fusions such as interferon α -2 α -1.

F. Chemokine Gene

The term "chemokine gene" refers to a nucleotide sequence, the expression of which in a cell produces a cytokine. The term chemokine refers to a group of structurally related low-molecular cytokines weight factors secreted by cells are structurally related having mitogenic, chemotactic or inflammatory activities. They are primarily cationic proteins of 70 to 100 amino acid residues that share four conserved cysteine residues. These proteins can be sorted into two groups based on the spacing of the two amino-terminal cysteines. In the first group, the two cysteines are separated by a single residue (C-x-C), while in the second group, they are adjacent (C-C). Examples of member of the 'C-x-C' chemokines include but are not limited to platelet factor 4 (PF4), platelet basic protein (PBP), interleukin-8 (IL-8), melanoma growth stimulatory activity protein (MGSA), macrophage inflammatory protein 2 (MIP-2), mouse Mig (m119), chicken 9E3 (or pCEF-4), pig alveolar macrophage chemotactic factors I and I (AMCF-I and -II), pre-B cell growth stimulating factor (PBSF), and IP10. Examples of members of the 'C-C' group include but are not limited to monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 2 (MCP-2), monocyte chemotactic protein 3 (MCP-3), monocyte chemotactic protein 4 (MCP-4), macrophage inflammatory protein 1 α (MIP-1- α), macrophage inflammatory protein 1 β (MIP-1- β), macrophage inflammatory protein 1 γ (MIP-1- γ), macrophage inflammatory protein 3- α (MIP-3- α), macrophage inflammatory protein 3 β (MIP-3- β), chemokine (ELC), macrophage inflammatory protein 4 (MIP-4), macrophage inflammatory protein 5 (MIP-5), LD78 β , RANTES, SIS-epsilon (p500), thymus and activation-regulated chemokine (TARC), eotaxin, I-309, human protein HCC-1/NCC-2, human protein HCC-3, mouse protein C10.

G. Pharmaceutical Protein Genes

The term "pharmaceutical protein gene" refers to nucleotide sequence, the expression of which results in the production of protein have pharmaceutically effect in the target cell. Examples of such pharmaceutical genes include the proinsulin gene and analogs (as described in PCT International Patent Application No. WO98/31397, growth hormone gene, dopamine, serotonin, epidermal growth factor, GABA, ACTH,

NGF, VEGF (to increase blood perfusion to target tissue, induce angiogenesis, PCT publication WO98/32859 published July 30, 1998), thrombospondin etc.

H. Pro-Apoptotic Genes:

- 5 The term "pro-apoptotic gene" refers to a nucleotide sequence, the expression thereof results in the programmed cell death of the cell. Examples of pro-apoptotic genes include p53, adenovirus E3-11.6K, the adenovirus E4orf4 gene, p53 pathway genes, and genes encoding the caspases. The p16 gene is also apoptotic in Rb positive, p16 negative, p53 wild type tumors. (Frizelle, *et al.* (1998) *Oncogene* 16:3087-95 and Sandig, *et al.* (1997) *Nature Medicine* 3:313)

10 I. Pro-Drug Activating Genes:

- The term "pro-drug activating genes" refers to nucleotide sequences, the expression of which, results in the production of protein capable of converting a non-therapeutic compound into a therapeutic compound, which renders the cell susceptible to killing by external factors or causes a toxic condition in the cell. An example of a
15 prodrug activating gene is the cytosine deaminase gene. Cytosine deaminase converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a potent antitumor agent. The lysis of the tumor cell provides a localized burst of cytosine deaminase capable of converting 5FC to 5FU at the localized point of the tumor resulting in the killing of many surrounding tumor cells. This results in the killing of a large number of tumor cells
20 without the necessity of infecting these cells with an adenovirus (the so-called bystander effect"). Additionally, the thymidine kinase (TK) gene (see e.g. Woo, *et al.* United States Patent No. 5,631,236 issued May 20, 1997 and Freeman, *et al.* United States Patent No. 5,601,818 issued February 11, 1997) in which the cells expressing the TK gene product are susceptible to selective killing by the administration of gancyclovir
25 may be employed.

J. Anti-Angiogenic Genes:

- The term "anti-angiogenic" genes refers to a nucleotide sequence, the expression of which results in the extracellular secretion of anti-angiogenic factors. Anti-angiogenesis factors include angiostatin, inhibitors of vascular endothelial growth factor
30 (VEGF) such as Tie 2 (as described in PNAS(USA)(1998) 95:8795-8800), and endostatin.

K. Modifications of Therapeutic Genes:

It will be readily apparent to those of skill in the art that modifications and or deletions to the above referenced genes so as to encode functional subfragments of the wild type protein may be readily adapted for use in the practice of the present invention.

- 5 The term "modifications" refers to changes in the genomic structure of the recombinant adenoviral vector. Such modifications include deletions and/or changes in amino acid coding sequence so as to produce a protein deficient in binding to its substrate.

For example, the reference to the p53 gene includes not only the wild type protein but also modified p53 proteins, allelic variations thereof, or proteins derived
10 from other mammalian species. The wild-type p53 sequence is well known in the art. Other mammalian p53 molecules are also known in the art and may be incorporated into the practice of the present invention such as murine p53, porcine p53, equine p53, bovine p53, canine p53, etc. The term "modified p53 proteins" refers to modifications to the primary, secondary, tertiary, or quaternary structure of the p53 protein which
15 retains the function of p53 proteins. Examples of such modified p53 proteins include modifications to p53 to increase nuclear retention, deletions such as the $\Delta 13-19$ amino acids to eliminate the calpain consensus cleavage site, modifications to the oligomerization domains (as described in Bracco, *et al.* PCT published application WO97/0492 or United States Patent No. 5,573,925). Alternatively, the p53 sequence
20 may be modified to replace the endogenous tetramerization domains with a leucine zipper oligomerization domain.

It will be readily apparent to those of skill in the art that the above therapeutic genes may be secreted into the media or localized to particular intracellular locations by inclusion of a targeting moiety such as a signal peptide or nuclear localization signal
25 (NLS). Also included in the definition of therapeutic transgene are fusion proteins of the therapeutic transgene with the herpes simplex virus type 1 (HSV-1) structural protein, VP22. Fusion proteins containing the VP22 signal, when synthesized in an infected cell, are exported out of the infected cell and efficiently enter surrounding non-infected cells to a diameter of approximately 16 cells wide. This system is particularly
30 useful in conjunction with transcriptionally active proteins (e.g. p53) as the fusion proteins are efficiently transported to the nuclei of the surrounding cells. See, e.g. Elliott, G. & O'Hare, P. Cell. 88:223-233:1997; Marshall, A. & Castellino, A.

Research News Briefs. Nature Biotechnology. 15:205:1997; O'Hare, *et al.* PCT publication WO97/05265 published February 13, 1997. A similar targeting moiety derived from the HIV Tat protein is also described in Vives, *et al.* (1997) J. Biol. Chem. 272:16010-16017.

5 V. Additional Modifications to the Virus:

The present invention also provides recombinant adenoviruses containing additional modifications to the viral genome such as targeting modifications, modifications to make the vectors replicate selectively in particular cell types or phenotypic states, controlled expression characteristics, suicide genes or additional
10 modifications to enhance cytotoxicity. However, this is not meant to imply that other modifications to the viral genome may not also be included or that multiple such modifications may not be employed.

A. Selectively Replicating:

The term "selectively replicating" refers to a recombinant viral vector capable of
15 preferential replication in one cell type versus another cell type, in a cell in one phenotypic state relative to another phenotypic state, or in a given cell type in response to an external stimuli. Selective replication is achieved by the use of replication control elements. The term "replication control elements" refers to DNA sequences inserted into the viral genome or modifications to the viral genome in order to produce
20 recombinant viral vectors which selectively replicate in one cell type versus another cell type, in a cell in one phenotypic state relative to another phenotypic state (cell state specific), or in a given cell type in response to an external stimuli (inducible). Examples of such replication control elements include cell-type specific promoter, cell state specific promoters, and inducible promoters.

25 1. Cell Type Specific Promoters:

Cell type specific replication may be achieved by the linkage of a cell type specific promoter to an early viral gene such as the E1, E1a, E2 or E4 gene when the virus is selected from the adenovirus genome. The term "cell type specific promoter" refers to promoters which are differentially activated in as a result of cell cycle
30 progression or in different cell types. Examples of cell-type specific promoters includes cell cycle regulatory gene promoters, tissue specific promoters or pathway responsive

promoters. In the preferred practice of the invention, the cell type specific promoter is linked to the E4 gene rather than the E1 gene because factors such as NF-IL6 can substitute for E1a in regulating E1a responsive promoters in the adenovirus in the absence of E1a function (Spergel, *et al.* (1992) J. Virol. 66:1021-1030).

5 a. Cell Cycle Regulatory Gene Promoters:

The term "cell cycle regulatory gene promoters" describe promoters for genes which are activated substantially upon entry into S-phase. Examples of such promoters include the E2F regulated promoters (e.g. DHFR, DNA polymerase alpha, thymidylate synthase, c-myc and b-myb promoters).

10 b. Tissue Specific Promoters:

Tissue specific promoters are well known in the art and include promoters active preferentially in smooth muscle (α -actin promoter), pancreas specific (Palmiter, *et al.* (1987) Cell 50:435), liver specific (Rovet, *et al.* (1992) J. Biol. Chem.. 267:20765; Lemaigne, *et al.* (1993) J. Biol. Chem.. 268:19896; Nitsch, *et al.* (1993) Mol. Cell. Biol. 13:4494), stomach specific (Kovarik, *et al.* (1993) J. Biol. Chem.. 268:9917, 15 pituitary specific (Rhodes, *et al.* (1993) Genes Dev. 7:913, prostate specific (United States Patent 5,698,443, Henderson, *et al.* issued December 16, 1997), etc.

 c. Pathway Responsive Promoters

The term "pathway-responsive promoter" refers to DNA sequences that bind a 20 certain protein and cause nearby genes to respond transcriptionally to the binding of the protein in normal cells. Such promoters may be generated by incorporating response elements which are sequences to which transcription factors bind. Such responses are generally inductive, though there are several cases where increasing protein levels decrease transcription. Pathway-responsive promoters may be naturally occurring or 25 synthetic. Pathway-responsive promoters are typically constructed in reference to the pathway or a functional protein which is targeted. For example, a naturally occurring p53 pathway-responsive promoter would include transcriptional control elements activated by the presence of functional p53 such as the p21 or bax promoter. Alternatively, synthetic promoters containing p53 binding sites upstream of a minimal 30 promoter (e.g. the SV40 TATA box region) may be employed to create a synthetic pathway-responsive promoter. Synthetic pathway-responsive promoters are generally

constructed from one or more copies of a sequence that matches a consensus binding motif. Such consensus DNA binding motifs can readily be determined. Such consensus sequences are generally arranged as a direct or head-to-tail repeat separated by a few base pairs. Elements that include head-to-head repeats (e.g. AGGTCATGACCT) are called palindromes or inverted repeats and those with tail-to-tail repeats are called everted repeats.

i. Examples Of Pathway Promoters:

Examples of pathway-responsive promoters useful in the practice of the present invention include synthetic insulin pathway-responsive promoters containing the consensus insulin binding sequence (Jacob, *et al.* (1995). *J. Biol. Chem.* 270:27773-27779), the cytokine pathway-responsive promoter, the glucocorticoid pathway-responsive promoter (Lange, *et al.* (1992) *J Biol. Chem.* 267:15673-80), IL1 and IL6 pathway-responsive promoters (Won K.-A and Baumann H. (1990) *Mol.Cell.Biol.* 10: 3965-3978), T3 pathway-responsive promoters, thyroid hormone pathway-responsive promoters containing the consensus motif: 5' AGGTCA 3', the TPA pathway-responsive promoters (TREs), TGF- β pathway-responsive promoters (as described in Grotendorst, *et al.* (1996) *Cell Growth and Differentiation* 7: 469-480). Additionally, natural or synthetic E2F pathway responsive promoters may be used. An example of an E2F pathway responsive promoter is described in Parr, *et al.* (1997), *Nature* 387:1145-1149 which describes an E2F-1 promoter containing 4 E2F binding sites and is reportedly active in tumor cells with rapid cycling. Examples of other pathway-responsive promoters are well known in the art and can be identified in the Database of Transcription Regulatory Regions on Eukaryotic Genomes accessible through the internet at <http://www.eimb.rssi.ru/TRRD>.

ii. Preferred Pathway Promoters:

In the preferred practice of the invention as exemplified herein, the vector comprises a synthetic TGF- β pathway-responsive promoter active in the presence of a functional TGF- β pathway such as the promoter containing SRE and PAI pathway responsive promoters. PAI refers to a synthetic TGF- β pathway-responsive promoter comprising sequences responsive to TGF- β signally isolated from the plasminogen activator-I promoter region. The PAI-pathway-responsive promoter may be isolated as a 749 base pair fragment isolatable from the plasmid p800luc (as described in

- Zonneveld, *et al.* (1988) PNAS 85:5525-5529 and available from GenBank under accession number J03836). SRE refers to a synthetic TGF- β response element comprising a repeat of 4 of the Smad-4 DNA binding sequences (GTCTAGAC as described in Zawel, *et al.* (1988) Mol. Cell 1:611-617. The SRE response element may
- 5 be generated by annealing complimentary oligonucleotides encoding the Smad-4 binding sequences and cloning in plasmid pGL#3 - promoter luciferase vector (commercially available from ProMega).

1. p53 Pathway Promoters:

- Similarly, a "p53 pathway-responsive promoter" refers to a transcriptional
- 10 control element active in the presence of a functional p53 pathway. The p53 pathway-responsive promoter may be a naturally occurring transcriptional control region active in the presence of a functional p53 pathway such as the p21 or mdm2 promoter. Alternatively, the p53 pathway-responsive promoter may be a synthetic transcriptional control region active in the presence of a functional p53 pathway such as the SRE and
- 15 PAI-RE pathway-responsive promoters. p53-CON describes a p53 pathway-responsive promoter containing a synthetic p53 response element constructed by insertion of two synthetic p53 consensus DNA binding sequences (as described in Funk, *et al.* (1992) Mol. Cell. Biol. 12:2866-2871) upstream of the SV40 TATA box. RGC refers to a synthetic p53 pathway-responsive promoter using a tandem of the p53
- 20 binding domains identified in the ribosomal gene cluster. p53CON and RGC response elements can be constructed by annealing complementary oligonucleotides and p53 responsive promoters can be constructed by cloning in plasmid pGL3-promoter luciferase vector (commercially available from ProMega).

d. Tissue Specific Promoters operably linked to an early gene.

- 25 Tissue specific promoters are well known in the art and include promoters active preferentially in smooth muscle (alpha-actin promoter), pancreas specific (Palmiter, *et al.* (1987) Cell 50:435), liver specific (Rovet, *et al.* (1992) J. Biol. Chem. 267:20765; Lemaigre, *et al.* (1993) J. Biol. Chem. 268:19896; Nitsch, *et al.* (1993) Mol. Cell. Biol. 13:4494), stomach specific (Kovarik, *et al.* (1993) J. Biol. Chem. 268:9917,
- 30 pituitary specific (Rhodes, *et al.* (1993) Genes Dev. 7:913 and prostate specific antigen promoter.

2. Cell State Specific:

Examples of different phenotypic states would include the neoplastic phenotype versus a normal phenotype in a given cell type. Selective replication is achieved by the use of viral replication control elements. The term viral replication control element refers to a DNA sequence engineered into the vector of the present invention such that the virus is preferentially enabled to replicate the viral genome in a particular type of target cell. Again, these cell state specific promoters may be linked to an early gene such as E1, E2, or preferably E4 to achieve selective replication in response to specific phenotypic states.

a. Tumor Specific:

In order to achieve expression of the adenovirus in tumor cells, one may employ a tumor specific promoter to drive expression of an early gene. The term "tumor specific promoters" refers to promoters which are active in tumor cells and inactive in cells which are not transformed. Examples of tumor specific promoters include the alpha-fetoprotein promoter, the tyrosinase promoter. The use of tumor specific promoters to achieve conditional replication of adenoviral vectors is described in co-pending United States Patent Application 08/433,798 filed May 3, 1995 and International Patent Application No. PCT/US96/06199 published as International Publication No. WO 96/34969 on November 7, 1996 the entire teaching of which is herein incorporated by reference.

For example, the alpha-fetoprotein promoter could be used to replace the endogenous E4 promoter and achieve greater selectivity in conditional replication. Other factors such as NF-IL6 can substitute for E1a in regulating E1a responsive promoters in the adenovirus in the absence of E1a function (Spergel, *et al.* (1992) J. Virol 66:1021-1030) and this can be avoided by substitution of the E4 promoter with a tumor specific promoter.

b. Repressor of Viral Replication:

Although one may use the pathway responsive promoter to drive replication of the virus in the presence of a functional pathway, alternatively, the one may use a pathway responsive promoter to drive expression of a repressor of viral replication to control expression. The term "repressor of viral replication" refers to a protein, if

expressed in a given cell, substantially represses viral replication. As will be appreciated by those of skill in the art, the repressor of viral replication will be dependent on the nature of the parent adenoviral vector from which the recombinant vector of the present invention is derived. For example, in the case of adenoviral
5 vectors or other DNA tumor viruses, the E2F-Rb fusion construct as described in European Patent Application No. 94108445.1 published December 6, 1995 (Publication number. 0 685 493 A1) may be employed. E2F-Rb fusion protein consists of the DNA binding and DP1 heterodimerizations domains of the human E2F transcription factor protein (amino acids 95-286 of wild type E2F) fused to the Rb
10 growth suppression domain (amino acids 379-928 of the wild type Rb protein). The E2F-Rb fusion protein is a potent repressor of E2F-dependent transcription and arrests cells in G1. The DNA binding domain is located at amino acids 128-193 and the dimerization domain is located at 194-289. The sequence of the human E2F-1 protein is available from GenBank under accession number M96577 deposited August 10,
15 1992. The sequences of E2F from other E2F family members of E2F from other species may be employed when constructing a vector for use in other species. In the situation where the recombinant virus is based on adenoassociated virus, the rep protein and its derivatives is an effective repressor of viral replication in the absence of adenovirus infection. In the situation where the virus is derived from herpes simplex
20 virus, the ICPO-NX, a deleted form of the immediate early protein ICPO (Liun, *et al.* (1998) J. Virol. 72:7785-7795), protein may be used as an effective repressor of viral replication. Similarly, any protein with dominant negative activity can be used as a repressor of viral replication.

These modifications may be combined with the cell cycle regulatory gene
25 promoters described above. For instance, an E2F pathway responsive promoter may be used to drive expression of the modified E1a coding sequence. Using a p53 pathway responsive promoter driving expression of E2F-Rb fusion protein, one achieves repression of both E1 function and E2 function because the E2F-Rb fusion protein will suppress both the E2 and E2F response elements. In p53 deficient tumor
30 cells, the p53 response element is inactive and E2F-Rb is not expressed. Consequently, E1a expression is enhanced by the presence of E2F in the tumor cell and the failure to repress E2 promoter enables viral replication to proceed.

3. Inducible Promoters

- The term "inducible promoter" refers to promoters which facilitate transcription of the therapeutic transgene preferable (or solely) under certain conditions and/or in response to external chemical or other stimuli. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, *et al.* (1996) J. Virol. 70(9):6054-6059; Hwang, *et al.* (1997) J. Virol 71(9):7128-7131; Lee, *et al.* (1997) Mol. Cell. Biol. 17(9):5097-5105; and Dreher, *et al.* (1997) J. Biol. Chem. 272(46): 29364-29371. Examples of radiation inducible promoters include those induced by ionizing radiation such as the Egr-1 promoter (as described in Manome, *et al.* (1998) Human Gene Therapy 9:1409-17; Takahashi, *et al.* (1997) Human Gene Therapy 8:827-833; Joki, *et al.* Human Gene Therapy (1995) 6:1507-1513; Boothman, *et al.* (1994) volume 138 supplement pages S68-S71; and Ohno, T (1995) Tanpakushitsu Kakusan Koso 40:2624-2630), X-ray inducible promoters such as the XRE promoter (as described in Boothman, *et al.* (1994) Radiation Research 138(Suppl.1):S68-S71), and UV inducible promoters such as those isolated from *Clostridium perfringens* (Garnier and Cole (1988) Mol. Microbiol. 2:607-614).

B. Alternative Modifications to the Viral Genome:

1. E1B Δ 55K deletion:

- As previously indicated the E1b 55K protein binds to p53. Consequently, in order to enhance the effect of the p53 introduced by the viral vector it is preferred to introduce Descriptions of E1B Δ 55K mutations to eliminate p53 binding described in McCormick, United States Patent No. 5,677,178 issued October 14, 1997, the entire teaching of which is herein incorporated by reference.
- In the preferred practice of the invention as exemplified herein, the virus is a recombinant adenoviral vector encoding p53 under control of the MLP promoter containing a deletion of nucleotides 2247-3272 of the adenoviral genome to eliminate the function of the E1b 55K protein.

2. E4 Modifications

- Additionally modifications to increase the potency of the vectors of the present invention include but are not limited to alterations within E1b. The vectors of the

- present invention may be modified to introduce mutations in E4 to increase the cytotoxicity (Muller, *et al.* (1992) *J. Virol.* 66:5867-5878) or contain upregulation of viral cytopathic proteins such as E4orf4 or E3 11.6K proteins. For example, the E4 region of the adenovirus genome has been implicated in viral DNA replication, host protein synthesis shut off and viral assembly. E4orf6 is sufficient for DNA replication and late protein synthesis in immortalized cells. However, E4orf6/7 appears to be required for replication in non-dividing cells. Consequently elimination of E4orf6/7 assists in restricting the replication of the virus in immortalized (i.e. tumor cells) and may be incorporated into the vectors of the present invention.
- Furthermore, E4 deletions have been shown to reduce the immunogenicity of the vectors (Wang, *et al.* (1997) *Gene Therapy* 4:393-400; Dedieu, *et al.* (1997) *J. Virol* 71:4626-37), but can affect the persistence of transgene expression depending on the open reading frames of E4 retained and the promoter used to drive expression of the transgene (Armentano, *et al.* (1997) *J. Virol* 71:2408-2416). The E4 region also encodes a protein (E4orf6) capable of binding to and inactivating the transcriptional activity of p53 (Dubner, *et al.* (1996) *Science* 272:1470-73). Therefore, it may be desirable to modify the E4 region to delete those open reading frames with undesirable properties for the particular virus construct while retaining those with desired properties. For example, for the conditionally replication virus described herein as the E1B Δ 55K-MLP-p53, the E4 orf6 region may be deleted to reduce inactivation of p53 while retaining E4orf3 to allow continued expression of p53 and replication of the virus. In order to preserve replication competency of the virus, either E4orf6 or E4orf3 should be retained.

- Examples of other E4 deleted adenoviral vectors are described in Gregory, *et al.*
- United States Patent No 5,670,488 issued September 23, 1997.

3. E1 deletions To Achieve Selective Replication in Rapidly Dividing Cells

- In order to achieved selective replication of adenovirus in rapidly dividing cells, the adenovirus may contain modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300 protein family members and one or more Rb protein family member protein but retain the transactivating function of the E1a CR3 domain and a deletion of the amino acids from

approximately 219 to approximately 289 of the E1a 289R protein (or approximately amino acids 173 to approximately amino acid 243 of the E1a 243R protein. In the preferred practice of the invention the deletion of the binding to the p300 family members is achieved by introducing a deletion corresponding to amino acids 4-25 of the E1a 243R and 289R proteins or amino acids 38-60 of the E1a 243R and 289R proteins. In the preferred practice of the invention the deletion of the binding to the pRb family members is achieved amino acids 111-123 of the E1a 243R and 289R proteins. Alternatively, deletion of the binding to the pRb family members may be achieved by eliminate of amino acids 124-127 of the E1a 243R and 289R proteins.

4. E3 Modifications:

The E3 region of the adenovirus encodes proteins which help adenovirally infected cells avoid clearance by the immune system (Wold, *et al.* (1995) *Curr. Top. Microbiol. Immunol.* 199:237-274). Upregulation of this region and subfragments thereof has been shown to prevent or decrease the immune response to virally infected cells, leading to longer term gene expression. (Ilan, *et al.* (1997) *PNAS* 94:2587:2592, Bunder, *et al.* (1997) *J. Virol.* 71:7623-28). Therefore, modifications to the E3 region (or sub-components thereof) to overexpress their proteins (e.g. by upregulating the E3 region using a strong constitutive promoter such as CMV) may be desirable to allow for a greater degree of viral replication due to its ability to avoid or delay the immune mediated clearance of infected cells.

B. Targeting Modifications:

The present invention provides recombinant viruses which contain "targeting modifications" in order to achieve preferential targeting of the virus to a particular cell type. The term "targeting modification" refers to modifications to the viral genome designed to result in preferential infectivity of a particular cell type. Cell type specificity or cell type targeting may also be achieved in vectors derived from viruses having characteristically broad infectivities such as adenovirus by the modification of the viral envelope proteins. For example, cell targeting has been achieved with adenovirus vectors by selective modification of the viral genome knob and fiber coding sequences to achieve expression of modified knob and fiber domains having specific interaction with unique cell surface receptors. Examples of such modifications are described in Wickham, *et al.* (1997) *J. Virol* 71(11):8221-8229 (incorporation of RGD peptides into

- adenoviral fiber proteins); Arnberg, *et al.* (1997) *Virology* 227:239-244 (modification of adenoviral fiber genes to achieve tropism to the eye and genital tract); Harris and Lemoine (1996) *TIG* 12(10):400-405; Stevenson, *et al.* (1997) *J. Virol.* 71(6):4782-4790; Michael, *et al.* (1995) gene therapy 2:660-668 (incorporation of gastrin releasing peptide fragment into adenovirus fiber protein); and Ohno, *et al.* (1997) *Nature Biotechnology* 15:763-767 (incorporation of Protein A-IgG binding domain into Sindbis virus). Other methods of cell specific targeting have been achieved by the conjugation of antibodies or antibody fragments to the envelope proteins (see, e.g. Michael, *et al.* (1993) *J. Biol. Chem.* 268:6866-6869, Watkins, *et al.* (1997) gene therapy 4:1004-1012; Douglas, *et al.* (1996) *Nature Biotechnology* 14: 1574-1578. Alternatively, particular moieties may be conjugated to the viral surface to achieve targeting (See, e.g. Nilson, *et al.* (1996) gene therapy 3:280-286 (conjugation of EGF to retroviral proteins).

VI. Experimental Results:

A. Vector Constructs:

- The E1B Δ 55K-MLP-p53 (FAMA) vector was prepared in substantial accordance with the teaching of Example 1 herein. Briefly, the adenovirus genome was modified by introducing a deletion of base pairs 2247 to 3272 of the adenovirus genome so that the ability of the 55K protein is unable to bind to and inhibit the transcriptional activity of p53. In substitution of this deletion was placed a polyA signal from adenovirus protein IX for the E1B19K upstream of the deletion and the cDNA encoding the Ad2 major late promoter and tripartite leader sequence driving expression of p53 from the plasmid pA/M/53 as describe in Wills, *et al.* (1994) *Human Gene Therapy* 5:1079-88. The remainder of the virus sequence is wild-type Ad5.

B. In vitro Experiments:

- The following experiments were performed to demonstrate that the major late promoter functioned in a temporal manner in expressing p53 from a replicating virus. Experiments were performed in substantial accordance with the teaching of Example 2 herein in order to evaluate the time course expression of p53 in various tumor cell lines. The data is presented in Figure 1-4 of the attached drawings. The data presented demonstrates the temporal expression of the cFAMA vector in a variety of tumor cell

lines with varying genotype as well as increased expression levels relative to replication deficient viruses expressing p53 (ACN53).

In one experiment, the expression of p53 from the FAMA vector was compared to a substantially similar vector wherein p53 was operably linked to the CMV

- 5 constitutive promoter. The experiment was conducted in substantial accordance with the teaching of Example 2.c. herein and the results are presented in Figure 4 of the attached drawings. his demonstrates that the FAMA virus does replicate in a temporal manner compared with the CMV containing FAIC virus.

- 10 In an additional expreiment conducted in accordance with Example 2.d., viral replication from FAMA was compared to the substantially similar FAIC vector where the temporal major late promoter was replaced by the constitutive CMV promoter. By expressing the p53 protein from the MLP promoter in a temporal manner, a greater replication of the virus is achieved relative to a constitutive promoter. The results are presented in Figure 5 of the attached drawings.

15 C. *In vivo* Experiments:

- In order to confirm the efficacy of the vectors of the present invention *in vivo* in an animal, the vectors of the present invention were evaluated in a mouse human prostate cancer model. The model and experiments were performed in substantial accordance with the teaching of Example 3 herein. The data is presented in Table 1
- 20 below and in Figure 6 of the attached drawings.

Table 1. Tumor Volume Following <i>In vivo</i> Administration of FAMA			
Virus	Avg. Tumor Volume (mm ³) \pm SD ¹	% T/C ²	# Animals Tumor Free
Saline control	1266 \pm 403	100	0/6
Wt Ad 5	16	1.3	5/6
cZAZA	39 \pm 13	3.1	3/6
cFAMA	25	2.0	5/6
cFAIC	16	1.3	5/6
FTCB	215 \pm 93	17	0/6
ZZCB	702 \pm 109	55	0/6

¹ Day 27 Post Initiation of Treatment

² Treated/saline control

- As can be seen from the data presented, the vectors of the present invention demonstrated antitumor efficacy *in vivo*. It should be noted that not only did the tumors fail to grow, but actually regressed in size such that 5 of 6 animals were tumor free 27 days post administration. This is particularly surprising in light of the non-replicating p53 vector FTCB in which p53 is also expressed from the CMV promoter. Also, the effect is not due to merely to the E1b55k deletion (as represented by cZAZA) which showed lower efficacy at 27 days post administration.

VII. Pharmaceutical Formulations:

- The present invention further provides a pharmaceutically acceptable formulation of the recombinant adenoviruses in combination with a carrier. The vectors of the present invention may be formulated for dose administration in accordance with conventional pharmaceutical practice with the addition of carriers and excipients. Dosage formulations may include intravenous, intratumoral, intramuscular, intraperitoneal, topical, matrix or aerosol delivery.

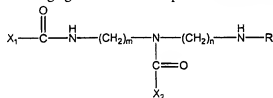
A. Carriers:

- The term "carrier" refers to compounds commonly used on the formulation of pharmaceutical compounds used to enhance stability, sterility and deliverability of the therapeutic compound. When the virus is formulated as a solution or suspension, the delivery system is in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorption monolaurate, triethanolamine oleate, etc.

B. Delivery Enhancing Agents:

The present invention further provides pharmaceutical formulations of the vectors of recombinant adenoviruses of the present invention with a carrier and a delivery enhancing agent(s). The terms "delivery enhancers" or "delivery enhancing agents" are used interchangeably herein and includes one or more agents which facilitate uptake of the virus into the target cell. Examples of delivery enhancers are described in co-pending United States Patent Application Serial No. _____ filed July 7, 1998. Examples of such delivery enhancing agents include detergents, alcohols, glycols, surfactants, bile salts, heparin antagonists, cyclooxygenase inhibitors, hypertonic salt solutions, and acetates. Alcohols include for example the aliphatic alcohols such as ethanol, N-propanol, isopropanol, butyl alcohol, acetyl alcohol. Glycols include glycerine, propyleneglycol, polyethyleneglycol and other low molecular weight glycols such as glycerol and thioglycerol. Acetates such as acetic acid, gluconic acid, and sodium acetate are further examples of delivery-enhancing agents. Hypertonic salt solutions like 1M NaCl are also examples of delivery-enhancing agents. Examples of surfactants are sodium dodecyl sulfate (SDS) and lysolecithin, polysorbate 80, nonylphenoxypolyoxyethylene, lysophosphatidylcholine, polyethyleneglycol 400, polysorbate 80, polyoxyethylene ethers, polyglycol ether surfactants and DMSO. Bile salts such as taurocholate, sodium tauro-deoxycholate, deoxycholate, chenodesoxycholate, glycocholic acid, glycochenodeoxycholic acid and other astringents such as silver nitrate may be used. Heparin-antagonists like quaternary amines such as protamine sulfate may also be used. Cyclooxygenase inhibitors such as sodium salicylate, salicylic acid, and non-steroidal antiinflammatory drug (NSAIDS) like indomethacin, naproxen, diclofenac may be used.

Delivery-enhancing agents includes compounds of the Formula I:



wherein n is an integer from 2-8, X₁ is a cholic acid group or deoxycholic acid group, and X₂ and X₃ are each independently selected from the group consisting of a cholic acid group, a deoxycholic acid group, and a saccharide group. At least one of X₂ and

X3 is a saccharide group. The saccharide group may be selected from the group consisting of pentose monosaccharide groups, hexose monosaccharide groups, pentose-pentose disaccharide groups, hexose-hexose disaccharide groups, pentose-hexose disaccharide groups, and hexose-pentose disaccharide groups.

- 5 The term "detergent" includes anionic, cationic, zwitterionic, and nonionic detergents. Exemplary detergents include but are not limited to taurocholate, deoxycholate, taurodeoxycholate, cetylpyridium, benalkonium chloride, Zwittergent3-14 detergent, CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate), Big CHAP, Deoxy Big CHAP, Triton-X-100 detergent, 10 C12E8, Octyl-B-D-Glucopyranoside, PLURONIC- F68 detergent, Tween 20 detergent, and TWEEN 80 detergent (CalBiochem Biochemicals).

- Unit dosage formulations of the present invention may be included in a kit of products containing the recombinant adenovirus of claim 1 in lyophilized form and a solution for reconstitution of the lyophilized product along with instructions for use.
- 15 Recombinant adenoviruses of the present invention may be lyophilized by conventional procedures and reconstituted.

- Another example of a delivery enhancing agent which may be employed in the formulations of present invention include calpain inhibitors. The "calpain inhibitor" (abbreviated "CI") refers to a compound which inhibits the proteolytic action of calpain- 20 I, e.g. μ -calpains. The term calpain inhibitors as used herein includes those compounds having calpain I inhibitory activity in addition to or independent of their other biological activities. A wide variety of compounds have been demonstrated to have activity in inhibiting the proteolytic action of calpains. Examples of calpain inhibitors are useful in the practice of the present invention include N-acetyl-leu-leu- 25 norleucinal also known as "calpain inhibitor I." Additional calpain inhibitors are described in the following United States Patents, herein incorporated by reference, United States Patent No. 5,716,980 entitled Alcohol or aldehyde derivatives and their use; United States Patent No. 5,714,471 entitled Peptide and peptide analog protease inhibitors; United States Patent No. 5,693,617 entitled Inhibitors of the 26s proteolytic 30 complex and the 20s proteasome contained therein; United States Patent No. 5,691,368 entitled Substituted oxazolidine calpain and/or cathepsin B inhibitors; United States Patent No. 5,679,680 entitled α -substituted hydrazides having calpain

- inhibitory activity; United States Patent No. 5,663,294 entitled Calpain-inhibiting peptide analogs of the kininogen heavy chain; United States Patent No. 5,661,150 entitled Drug for neuroprotection; United States Patent No. 5,658,906 entitled Cysteine protease and serine protease inhibitors; United States Patent No. 5,654,146 entitled
- 5 Human ice homolog; United States Patent No. 5,639,783 entitled Ketone derivatives; United States Patent No. 5,635,178 entitled Inhibition of complement mediated inflammatory response using monoclonal antibodies specific for a component forming the C5b-9 complex which inhibit the platelet or endothelial cell activating function of the C5b-9 complex; United States Patent No. 5,629,165 Neural calcium-activated neutral
- 10 proteinase inhibitors; United States Patent No. 5,622,981 entitled Use of metabotropic receptor agonists in progressive neurodegenerative diseases; United States Patent No. 5,622,967 entitled Quinolone carboxamide Calpain inhibitors; United States Patent No. 5,621,101 entitled Protein kinase inhibitors for treatment of neurological disorders; United States Patent No. 5,554,767 entitled Alpha-mercaptoacrylic acid derivatives
- 15 having calpain inhibitory activity; United States Patent No. 5,550,108 entitled Inhibition of complement mediated inflammatory response; United States Patent No. 5,541,290 entitled Optically pure calpain inhibitor compounds; United States Patent No. 5,506,243 entitled Sulfonamide derivatives; United States Patent No. 5,498,728 entitled Derivatives of L-tryptophanal and their use as medicinals; United States Patent
- 20 No. 5,498,616 entitled Cysteine protease and serine protease inhibitors; United States Patent No. 5,461,146 entitled Selected protein kinase inhibitors for the treatment of neurological disorders; United States Patent No. 5,444,042 entitled Method of treatment of neurodegeneration with calpain inhibitors; United States Patent No. 5,424,325 entitled aminoketone derivatives; United States Patent No. 5,422,359
- 25 entitled α -aminoketone derivatives; United States Patent No. 5,416,117 entitled Cyclopropanone derivatives; United States Patent No. 5,395,958 entitled Cyclopropene derivatives; United States Patent No. 5,340,922 entitled Neural calcium-activated neutral proteinase inhibitors; United States Patent No. 5,336,783 entitled Calpain inhibitor cystamidin A and its production; United States Patent No. 5,328,909
- 30 entitled Cyclopropanone derivatives; and United States Patent No. 5,135,916 entitled Inhibition of complement mediated inflammatory response. The uses of calpain inhibitors in gene therapy protocols is further described in Atencio, *et al.*, co-pending United States Patent Applications Serial Nos. 09/172,685 and 60/104,321 filed October

15, 1998. In the preferred practice of the invention, the calpain inhibitor is N-acetyl-leu-leu-norcinal (calpain inhibitor-1) commercially available from Boehringer-Mannheim (Indianapolis, IN).

IIX. Therapeutic Applications:

5 A. Anti-Neoplastic Applications:

The present invention provides a method of eliminating neoplastic cells in a mammalian organism by the administration of a recombinant viral vector of the present invention to a neoplastic cell. The term "neoplastic cell" is a cell displaying an aberrant growth phenotype characterized by independence of normal cellular growth controls.

- 10 As neoplastic cells are not necessarily replicating at any given time point, the term neoplastic cells comprise cells which may be actively replicating or in a temporary non-replicative resting state (G1 or G0). Localized populations of neoplastic cells are referred to as neoplasms. Neoplasms may be malignant or benign. Malignant neoplasms are also referred to as cancers. The term cancer is used interchangeably
- 15 herein with the term tumor. Neoplastic transformation refers the conversion of a normal cell into a neoplastic cell, often a tumor cell. The term "mammalian organism" includes, but is not limited to, humans, pigs, horses, cattle, dogs, cats.

- The present invention provides a method of ablating neoplastic cells in a mammalian organism *in vivo* by the administration of a pharmaceutically acceptable
- 20 formulation of the recombinant adenovirus described above. The term "ablating" means the substantial reduction of the population of viable neoplastic cells so as to alleviate the physiological maladictions of the presence of the neoplastic cells. The term "substantial" means a reduction in the population of viable neoplastic cells in the mammalian organism by greater than approximately 20% of the pretreatment
- 25 population. The term "viable" means having the uncontrolled growth and cell cycle regulatory characteristics of a neoplastic cell. The term "viable neoplastic cell" is used hereing to distinguish said cells from neoplastic cells which are no longer capable of replication. For example, a tumor mass may remain following treatment, however the population of cells comprising the tumor mass may be dead. These dead cells have
- 30 been ablated and lack the ability to replicate, even though some tumor mass may remain.

In the preferred practice of the invention as exemplified herein, a recombinant adenovirus containing a deletion of the E1B-55K gene function and expressing a tumor suppressor gene from the adenoviral major late promoter is formulated with a pharmaceutically acceptable carrier for administration by intravenous, intraperitoneal, or intratumor injection. The appropriate dose and method of administration of the vector to be administered to the mammalian organism in need of treatment will be determined by the skilled artisan taking into account the extent of metastasis of the primary tumor, the delivery enhancer(s) included in the formulation, the extent to which the immunological response is suppressed, etc. Each of these latter factors will decrease the dosage of the vector provided to the mammalian organism in need of treatment. In the preferred practice of the invention, a dosage of approximately 1×10^5 to 1×10^{13} particles (preferably 1×10^6 to 1×10^{11} particles, most preferably 1×10^7 to 1×10^{10} particles) will be administered to the mammalian organism in one or more dosages in a treatment regimen. The typical course of treatment will be the daily administration of a pharmaceutically acceptable formulation of the vector of the present invention over a period of three to ten days, preferably five to eight days. In a preferred embodiment, the tumor suppressor gene is the wild-type p53 gene. In another preferred embodiment, the tumor suppressor gene encodes is a VP22-p53 fusion protein.

In a further preferred practice of the invention, the pharmaceutically acceptable carrier contains a delivery enhancing agent. In a further preferred practice of the invention, the delivery enhancing agent is a calpain inhibitor. In the most preferred practice of the invention as exemplified herein, the recombinant adenoviral vector E1B-Δ55K-MLP-p53 is formulated in a carrier solution further comprising the calpain inhibitor n-acetyl-leu-leu-norcinal (calpain inhibitor 1) at a concentration of from approximately 1 to 50 micromolar. In such instances, the daily dosage may be reduced as compared to a formulation absent such delivery enhancing agents by a factor of one to two logs.

Preferably one employs an adenoviral vector endogenous to the mammalian type being treated. Although it is generally favored to employ a virus from the species to be treated, in some instances it may be advantageous to use vectors derived from different species which possess favorable pathogenic features. For example, it is reported (WO 97/06826 published April 10, 1997) that ovine adenoviral vectors may be used in

human gene therapy to minimize the immune response characteristic of human adenoviral vectors. By minimizing the immune response, rapid systemic clearance of the vector is avoided resulting in a greater duration of action of the vector.

While the present invention provides a method of use of the recombinant
5 adenoviruses alone, the recombinant adenoviruses of the present invention and formulations thereof may be employed in combination with conventional chemotherapeutic agents or treatment regimens. Examples of such chemotherapeutic agents include inhibitors of purine synthesis (e.g., pentostatin, 6-mercaptopurine, 6thioguanine, methotrexate) or pyrimidine synthesis (e.g. Pala, azarbine), the
10 conversion of ribonucleotides to deoxyribonucleotides (e.g. hydroxyurea), inhibitors of dTMP synthesis (5-fluorouracil), DNA damaging agents (e.g. radiation, bleomycines, etoposide, teniposide, dactinomycin, daunorubicin, doxorubicin, mitoxantrone, alkylating agents, mitomycin, cisplatin, procarbazine) as well as inhibitors of microtubule function (e.g. vinca alkaloids and colchicine) and farnesyl protein
15 transferase inhibitors. Chemotherapeutic treatment regimens refers primarily to non-chemical procedures designed to ablate neoplastic cells such as radiation therapy. In such instances, the daily dosage in the course of treatment is reduced in comparison to those dosages provided absent such chemotherapeutic agents.

It has been observed that the immune system is capable of recognizing and
20 eliminating recombinant viral vectors. As this would effectively reduce the amount of adenovirus reaching the target cell, it is preferable in many instances to administer the compounds of the present invention in combination with immunosuppressive agents such as etoposide. In the preferred practice of the invention, the immunosuppressive agent is administered in advance, preferably for about a week in advance of the
25 introduction of the recombinant viral vector of the present invention to eliminate the humoral immune response to the viral particles. In the preferred practice of the invention, a pharmaceutically acceptable formulation of the vector of the present invention is administered intratumorally following the administration of a immunosuppressive agent for a period of from about one day to about two weeks in
30 advance of administration of the vector. The vector is preferably an adenoviral vector and further contains a deletion of the E1B-55K protein and contains an expression cassette expressing the p53 tumor suppressor gene from an adenoviral major late

- promoter. In the preferred practice of the invention, the immunosuppressive agent is etoposide and is administered daily for a period of from about 1 to 7 days (preferably 3-7 days) prior to administration of the vector. In such instances, the daily dosage in the course of treatment is reduced in comparison to those dosages provided absent such immunosuppressive agents.

- The present invention also provides a method of ablating neoplastic cells in a population of normal cells contaminated by said neoplastic cells *ex vivo* by the administration of a recombinant adenovirus of the present invention to said population. An example of the application of such a method is currently employed in *ex vivo* applications such as the purging of autologous stem cell products commonly known as bone marrow purging. The term "stem cell product" refers to a population of hematopoietic, progenitor and stem cells capable of reconstituting the long term hematopoietic function of a patient who has received myoablative therapy. Stem cell products are conventionally obtained by apheresis of mobilized or non-mobilized peripheral blood. Apheresis is conventionally achieved through the use of known procedures using commercially available apheresis apparatus such as the COBE Spectra Apheresis System, commercially available from COBE International, 1185 Oak Street, Lakewood, CO. It is preferred that treatment conditions be optimized to achieve a "3-log purge" (i.e. removal of approximately 99.9% of the tumor cells from the stem cell produce) and most preferably a "5-log purge" (removal of approximately 99.999% of tumor cells from the stem cell product). In the preferred practice of the invention, a stem cell product of 100 ml volume would be treated at a concentration of from about 1×10^6 to 1×10^{10} particles/ml of the recombinant adenovirus of the present invention for a period of approximately 4 hours at 37°C.

B. Recruitment of Dendritic Cells:

- The present invention provides a recombinant viral vectors capable of recruiting immature dendritic cells to a tumor site and exposing the dendritic cells to a localized high concentration of tumor antigens characteristic of the tumor present in the patient. The vectors of the present invention are specifically engineered to induce killing of tumor cells. The lysed tumor cell (or the apoptotic bodies produced by an apoptosed tumor cell) provides a rich localized concentration of tumor specific proteins. By introducing a gene encoding a dendritic cell chemoattractant, immature dendritic cells

capable engulfing tumor antigens are recruited to the site of the lysed tumor cells thereby engulfing tumor antigens and presenting these antigens to the immune system. The term "dendritic cell chemoattractants" refers to chemotactic chemokines capable of attracting and/or directing the migration of dendritic cells to a particular location. It has been demonstrated that certain chemokines, fMLP (representative of formyl peptides of bacterial origin), C5a and the C-C chemokines monocyte chemoattractant protein (MCP)-3, macrophage inflammatory protein (MIP)-1 alpha/LD78, and RANTES, have been involved in the recruitment and chemotactic migration of dendritic cells. Sozzani, *et al.* (1995) J. Immunol. 155(7):3292-5. Xu, *et al.* suggest that all C-C chemokines, including MCP-1, MCP-2, MCP-3, MIP1 alpha, MIP-1 beta, and RANTES, induced migration of DC-enriched cells cultured with or without IL-4. Xu, *et al.* (1996) J. Leukoc. Biol. 60(3):365-71. Greaves, *et al.* (1997) J. Exp. Med. 186(6):837-44, indicate that MIP-3-alpha specifically interacts with the CC chemokine receptor 6 expressed on dendritic cells capable of directing migration of dendritic cells. In the preferred practice of the invention, the dendritic cell chemoattractant is MIP-3-alpha. The dendritic cell chemoattractant may be expressed intracellular form where it is released upon cell lysis or in secreted form by the use of a signal peptide. Upon expression of the dendritic cell chemoattractant, the dendritic cells then engulf the tumor antigens or apoptotic bodies, mature and migrate through existing pathways to the lymph and present the tumor antigens to the T-cells. The resulting T-cells are then capable of recognizing and killing tumor cells. The appropriate dosage regimen in such instances is in accordance with the typical anti-neoplastic dosage formulation and regimen as described above.

IX. Diagnostic Applications

In addition to therapeutic applications described above, the vectors of the present invention are also useful for diagnostic purposes. For example, the vectors of the present invention may incorporate a reporter gene in place of the therapeutic gene which is expressed upon viral replication. The term "reporter gene" refers to a gene whose product is capable of producing a detectable signal alone or in combination with additional elements. Examples of reporter genes includes the beta-galactosidase gene, the luciferase gene, the green fluorescent protein gene, nucleotide sequences encoding proteins detectable by imaging systems such as X-rays or magnetic field imaging

systems (MRI). Alternatively, such vectors may also be employed to express a cell surface protein capable of recognition by a binding molecule such as a fluorescently labelled antibody. Examples of *in vivo* applications include imaging applications such as X-ray, CT scans or Magnetic Resonance Imaging (MRI).

5 X. Method of Making The Compositions:

The present invention further provides a method of producing the recombinant adenovirus comprising the modifications to packaging domains described above, said method comprising the steps of:

- a. infecting a producer cell with a recombinant virus;
- 10 b. culturing said infected producer cell under conditions so as to permit replication of the viral genome in the producer cell;
- c. harvesting the producer cells, and
- d. purifying the recombinant adenovirus.

The term "infecting" means exposing the recombinant adenovirus to the
15 producer cell under conditions so as to facilitate the infection of the producer cell with the recombinant virus. In cells which have been infected by multiple copies of a given virus, the activities necessary for viral replication and virion packaging are cooperative. Thus, it is preferred that conditions be adjusted such that there is a significant probability that the producer cells are multiply infected with the virus. An example of a
20 condition which enhances the production of virus in the producer cell is an increased virus concentration in the infection phase. However, it is possible that the total number of viral infections per producer cell can be overdone, resulting in toxic effects to the cell. Consequently, one should strive to maintain the infections in the virus concentration in the range of 10^6 to 10^{10} , preferably about 10^9 , virions per ml.
25 Chemical agents may also be employed to increase the infectivity of the producer cell line. For example, the present invention provides a method to increase the infectivity of producer cell lines for viral infectivity by the inclusion of a calpain inhibitor. Examples of calpain inhibitors useful in the practice of the present invention include calpain inhibitor 1 (also known as N-acetyl-leucyl-leucyl-norleucinal, commercially available
30 from Boehringer Mannheim).

- The term "producer cell" means a cell capable of facilitating the replication of the viral genome of the recombinant adenovirus to be produced and capable of complementing the packaging defects of the recombinant adenovirus. A variety of mammalian cell lines are publicly available for the culture of recombinant adenoviruses.
- 5 For example, the 293 cell line (Graham and Smiley (1977) J. Gen. Virol. 36:59-72) has been engineered to complement the deficiencies in El function and is a preferred cell line for the production of the current vectors. In a similar manner, cell lines may be developed incorporating viral sequences stably integrated into the viral genome. For example, Cunningham and Davidson ((1997, Virol 197:116-124) demonstrate that
- 10 overlapping cosmids may be used to complement deleted viral functions for herpes viral vectors. A similar approach may be employed to complement adenoviral and other viral elements. Additional genes, such as those encoding drug resistance, can be included to allow selection or screening for the presence of the recombinant complementing vector. Such additional genes can include, for example, genes encoding neomycin resistance,
- 15 multi-drug resistance, thymidine kinase, beta-galactosidase, dihydrofolate reductase (DHFR), and chloramphenicol acetyl transferase. Examples of other producer cells parent cell lines which may be employed include HeLa cells, PERC.6 cells (as described in publication WO/97/00326, application serial No. PCT/NL96/00244 and the A549-E1 cell line (as described in International Patent Application No.
- 20 PCT/US97/810039 published February 23, 1998 as International Publication No. WO98/US3473.

- The term "culturing under conditions to permit replication of the viral genome" means maintaining the conditions for the infected producer cell so as to permit the virus to propagate in the producer cell. It is desirable to control conditions so as to maximize
- 25 the number of viral particles produced by each cell. Consequently it will be necessary to monitor and control reaction conditions such as temperature, dissolved oxygen, pH, etc. Commercially available bioreactors such as the CeliGen Plus Bioreactor (commercially available from New Brunswick Scientific, Inc. 44 Talmadge Road, Edison, NJ) have provisions for monitoring and maintaining such parameters.
- 30 Optimization of infection and culture conditions will vary somewhat, however, conditions for the efficient replication and production of virus may be achieved by those of skill in the art taking into considerations the known properties of the producer cell line, properties of the virus, type of bioreactor, etc. When 293 cells are employed as

the producer cell line, oxygen concentration is preferably maintained from approximately 50% to approximately 120% dissolved oxygen, preferably 100% dissolved oxygen. When the concentration of viral particles (as determined by conventional methods such as HPLC using a Resource Q column) begins to plateau, the reactor is harvested.

The term "harvesting" means the collection of the cells containing the recombinant adenovirus from the media. This may be achieved by conventional methods such as differential centrifugation or chromatographic means. At this stage, the harvested cells may be stored or further processed by lysis and purification to isolate the recombinant virus. For storage, the harvested cells should be buffered at or about physiological pH and frozen at -70C.

The term "lysis" refers to the rupture of the producer cells. Lysis may be achieved by a variety of means well known in the art. When it is desired to isolate the viral particles from the producer cells, the cells are lysed, using a variety of means well known in the art. For example, mammalian cells may be lysed under low pressure (100-200 psi differential pressure) conditions or conventional freeze thaw methods. Exogenous free DNA/RNA is removed by degradation with DNase/RNase.

The term "purifying" means the isolation of a substantially pure population of recombinant virus particles from the lysed producer cells. Conventional purification techniques such as chromatographic or differential density gradient centrifugation methods may be employed. In the preferred practice of the invention, the virus is purified by column chromatography in substantial accordance with the process of Huyghe *et al.* (1995) *Human Gene Therapy* 6: 1403-1416 as described in co-pending United States Patent application Serial No. 08/400,793 filed March 7, 1995.

Additional methods and procedures to optimize production of the recombinant adenoviruses of the present invention are described in co-pending United States Patent Application Serial No. 09/073,076, filed May 4, 1998.

The purified virus is then admixed with appropriate excipients and carriers or delivery enhancing agents. The solution is sterilized for individual packaging and vialled for storage.

- Alternatively, the virus may be lyophilized for storage and reconstituted in a solution containing delivery enhancing agents, buffers, preservatives, cryoprotectants and/or carriers. The lyophilized virus and the reconstitution solution may be packaged together as a kit for consumption by the end user along with instructions for appropriate handling and administration.

EXAMPLES

- As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed below, without departing from the spirit or essential characteristics of the invention.
- 10 The particular embodiments of the invention described below, are, therefore to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples provided below.

Example 1. Construction of E1Bd155K-MLP-p53 (cFAMA)

- The E1B55K-MLP-p53 (cFAMA) adenovirus was prepared using the
- 15 oligonucleotide site directed mutagenesis technique of Deng and Nickoloff (1992) *Anal. Biochem* 200, 81-88. All of the reagents, bacterial strains, and vectors used for mutagenesis were provided in the Transformer Site-Directed mutagenesis kit (commercially available from Clontech, Palo Alto, CA). The Ad5 region containing the sequence to be mutated was inserted into Clontech plasmid pEGFP-1 and named pXB-
- 20 E1B. Two primers were designed to anneal to: (1) Ad5 sequence 2236 to 2260 and modify G2247 to thymidine and T2248 to cytosine and (2) Ad5 sequence 3255 to 3284 and modify T3272 to cytosine. A third primer was designed to eliminate the HindIII site within the original vector sequence, and provide a method for selection.

- For the mutagenesis reaction, the mutagenic oligonucleotides were first
- 25 phosphorylated at the 5' end, and then annealed to denatured pXB-E1B template DNA. The annealed primer/template reactions were incubated with T4 DNA polymerase, T4 DNA ligase and deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) to synthesize a complementary mutant strand. The complementary strand synthesis reaction was then transformed into the bacterial strain, BMH 71-18 *mutS*. This bacterial strain is defective
- 30 for mis-match repair, preventing undesired repair of the mutant strand. The transformants were digested with HindIII to cut any parental plasmid strands, and retransformed into DH5 α for amplification. Potential E1B mutants were then screened

by restriction enzyme analysis to confirm the desired mutations.

This procedure was used to introduce restriction enzyme nuclease cleavage sites in the E1B 55K coding region. The first site was introduced by modifying positions 2247 and 2248 of the wild type Ad5 genome wherein a guanine²²⁴⁷ was replaced with a thymidine and thymidine²²⁴⁸ replaced with cytosine (respectively) to introduce a EcoRI cleavage site. This results in a modification of the E1B coding sequence at position 77 from valine to serine. A second restriction site was introduced at position 3272 wherein thymidine³²⁷² was replaced with cytosine site (silent mutation) to introduce an XhoI site. The new restriction enzyme sites were used in a restriction enzyme digest with EcoRI and XhoI.

A cassette containing the p53 coding sequence, under control of the adenovirus Major Late Promoter and tripartite leader sequence, was removed by EcoRI and partial XhoI digestion from the plasmid, pAd-MLP-p53. This plasmid is based on the pBR322 derivative pML2 (pBR322 deleted for base pairs 1140-2490) and contains an adenovirus type 5 sequences extending from base pair 1 to 5788 except that it is deleted for adenovirus type 5 base pairs 357-3327. At this site the Ad5 357-3327 deletion, a transcription unit is inserted which is comprised of the adenovirus type 2 major late promoter, the adenovirus type 2 tripartite leader DNA and human p53 cDNA. This EcoRI/XhoI fragment is inserted into the EcoRI and XhoI sites introduced into the E1B55k coding region. The polyA sequence that follows wtAd5 pIX was amplified by PCR (Ad5 sequence 4001-4368). The primers used for amplification included sequences to introduce an EcoRI site at the 5' end of the Ad5 sequence, and a SacII site at the 3' end of the Ad5 sequence. This fragment was then inserted into the EcoRI-SacII sites immediately following the E1B19k coding sequence. The SacII site was included in the MLP-p53 cassette inserted previously, and was upstream of the MLP promoter sequence. The resulting E1B mutation results in a sequence encoding the first 76 amino acids of the E1B55K protein followed by 11 missense amino acids resulting in a non-functional deleted E1B protein.

Construction of the E1BΔ55K-MLP-p53 adenovirus was carried out by using homologous recombination in the adenovirus E1-region containing 293 cell line by the method of McGory, *et al.*, (1988) Virology 163, 614-617. This method requires two fragments of DNA, one a transfer plasmid containing the E1B55k deleted/MLP-p53 cassette and the other Ad5 viral DNA containing the wtAd5 genome ("Ad5 large

- fragment"), from Ad5 bp918 to the 3' ITR (bp 35935). The transfer plasmid used, pXC1-E1B55-2A, contains wtAd5 sequences from 22-2246. For recombination to produce adenovirus cFAMA, the viral large fragment and pXC1-E1B53-2A were cotransfected into 293 cells by calcium phosphate mediated transfection. After 5 hours the precipitate was rinsed from the cells and normal media replaced. At 15 days after the initial transfection, viral "comets" were isolated, plaque purified two times, and subsequently viral DNA was screened using restriction enzyme analysis and DNA sequencing. Viral stocks were purified by double cesium chloride gradients and quantitated by column chromatography as described in Huyghe, *et al.* (1995) Human Gene Therapy 6:1403-1416.

Example 2. *In vitro* Evaluation of E1Bdl55K-MLP-p53 (FAMA)

Example 2.a. p53 Expression MRC9 Cells

- Levels of p53 expression from two different viral constructs (E1Bdl55K-MLP-p53 (cFAMA) and ACN53 (Wills, *et al.* (1994) Human Gene Therapy, *supra*)) were evaluated in MRC9. Six well plates were seeded with 6×10^5 MRC9 cells per well. Infection was performed for a one hour pulse in a volume of 250 microliters at two different concentrations, 1.8×10^8 particles/ml and 1.8×10^9 particles/ml. The cells were harvested and assayed using a BioRad Catalog Number 500-0006 Kit in substantial accordance with the instructions provided by the manufacturer. The cells were harvested by scraping at 24, 48, and 72 hours as indicated. The cells were resuspended in lysis buffer (50mM Tris, 250 mM NaCl, 0.1% NP40, 50 mM NaF, 5 mM EDTA) and lysed by the freeze thaw method (repeated three times). The cells were stained with the BioRad dye reagent concentrate provided with the kit and the absorbance was determined at a wavelength of 595 nm. The primary antibody was a murine anti-p53 antibody (commercially available from Novacastra as catalog number 1801 diluted 1:1000). The secondary antibody was Goat-anti-Mouse HRP (commercially available from Amersham as catalog number NA931). The data is presented in Figure 1 of the attached drawings. As can be seen from the data presented, the replication competent MLP-p53 construct resulted in expression later in time than the replication deficient CMV-p53 virus (ACN53).

Example 2.b. p53 Expression SK-HEP1 Cells

This evaluation was performed in substantial accordance with the teaching of Example 2.a above except that the experiment was conducted in SK-HEP1 hepatocellular carcinoma cells and the six well plate was seeded with 7.5×10^5 cells per well. The results are presented in Figure 2 of the attached drawings.

Example 2.c. p53 Expression NCI H358 Cells

This evaluation was performed in substantial accordance with the teaching of Example 2.a above except that the experiment was conducted in NCI H358 cells except that the six well plate was seeded with 1.5×10^5 cells per well. The cells were infected as in Example 2.a. above using 1.8×10^8 particles/ml concentration of the indicated viruses for Figure 3 and 1.8×10^8 for Figure 4. The results are presented in Figure 3 and 4 of the attached drawings.

Example 2.d. Time Course of Viral Replication

- SK-BR3 cells were infected with a one hour pulse of the following recombinant adenoviral vectors at a concentration of 1.8×10^8 particles/ml:
1. Mock: non-infected cells
 2. rAdcon: a recombinant adenovirus lacking E1 and protein IX function without a p53 coding sequence (Wills, et al.)
 3. E1B Δ 55K: A recombinant adenovirus containing the E1B-55K deletion described in Example 1 above with no exogenous transgene cassette.
 4. 55K/MLPp53: The recombinant adenovirus cFAMA prepared in substantial accordance with the teaching of Example 1 above.
 5. rAd-p53: ACN53 (Wills, et al.)
 6. 55K/CMVp53: A recombinant adenovirus cFAIC containing the E1B-55K deletion described above further comprising an expression cassette encoding p53 under control of the CMV promoter
 7. Ad5WT: Wild type adenovirus type 5.

The cells were harvested at approximately 48 hours post infection. The DNA was applied to a agarose gel and stained with ethidium bromide according to techniques well known in the art. The results are presented in Figure 5 of the attached drawings.

Example 3. Demonstration of Therapeutic Efficacy *In vivo*

PC-3 cells (prostate carcinoma, p53 null) were injected subQ into flanks of nude mice. When tumors were palpable (day 11), virus was intratumorally injected for 5 consecutive days at a dose of 1×10^{10} particles per injection on days 11-15 post PC-3

cell injection. The six different viral constructs cFAMA, cFAIC, FTCB, ZZCB, cZAZA and wildtype Ad5 (described in Example 2 above) were compared. V-PBS refers to phosphate buffered saline containing 3% sucrose and 2mM magnesium chloride.

5 Tumor volume was evaluated on days 1, 4, 7, 11, 15, 19, 23, and 27. The results of the evaluations are presented in Figure 5 of the accompanying drawings and presented in the Table 1 above.

Example 4. Construction of E1B Δ 55K-MLP-Cytosine Deaminase

The E1B Δ 55K-MLP-Cytosine Deaminase vector is prepared in substantial accordance with the teaching of Example 1 herein. However, the transfer plasmid
10 containing the p53 coding sequence is replaced with a DNA sequence encoding the cytosine deaminase gene.

Example 5. Construction of AFP-E4-E1B Δ 55K-MLP-p53

The AFP-E4-E1B Δ 55K-MLP-p53 is a recombinant adenovirus which is similar to the E1B Δ 55K-MLP-p53 vector is prepared in substantial accordance with the
15 teaching of Example 1 herein. However, in this construction the E4 gene is operably linked to the alpha-fetoprotein tumor specific promoter sequence (AFP) facilitating the replication of this virus in hepatocellular carcinoma cells. Other factors such as NF-IL6 can substitute for E1a in regulating E1a responsive promoters in the adenovirus in the absence of E1a function (Spergel, *et al.* (1992) J. Virol 66:1021-1030) and this can be
20 avoided by substitution of the E4 promoter with a tumor specific promoter.

Example 6. Construction of AFP-E4-E1B Δ 55K-MLP-IFN2a

The AFP-E4-E1B Δ 55K-MLP-IFN2a describes a recombinant adenovirus which is similar to the AFP-E4-E1B Δ 55K-MLP-p53 vector is prepared in substantial
25 accordance with the teaching of Example 5 herein. However, in this construction the p53 gene is replaced with the DNA sequence encoding interferon alpha-2b.

Example 7. Construction of E1A Δ 01/07-E1B Δ 55K-MLP-p53

The E1A Δ 01/07-E1B Δ 55K-MLP-p53 is a recombinant adenovirus which is substantially similar to the E1B Δ 55K-MLP-p53 vector is prepared in substantial
30 accordance with the teaching of Example 1 herein. However, in this virus the E1a gene is modified to contain the in-frame deletion mutations Δ 1101 and Δ 1107, as described in Jelsma *et al.*, (1998) Virology 163, 494-502 and are constructed using the

- oligonucleotide site directed technique of Zoeller and Smith (1984) *DNA* 3, 479-488, as modified by Kunkel (1985) *PNAS* 82, 488-492. All of the reagents, bacterial strains, and M13 vectors used for mutagenesis are provided in the Muta-Gene *in vitro* mutagenesis kit (commercially available from Bio Rad, Hercules, CA). The M13
- 5 template DNA, useful for mutagenesis of the E1A region, contains Ad5 sequences from nucleotide positions 22-1339 inserted between the BamHI and XbaI restriction enzyme sites in the multiple cloning sequence of M13mp19. The resulting bacteriophage construct, M13mp19E1A, is then propagated in *dut ung E. coli* bacterial strain CJ236 which results in an occasional incorporation of uracil in place of thymidine in the newly
- 10 synthesized DNA. The oligonucleotides, for construction of the E1 mutants, are synthesized to consist of sequences of either 11 or 12 nucleotides of Ad5 sense DNA on either side of the sequence that was to be removed.

- For the mutagenesis reaction, the mutagenic oligonucleotides are first phosphorylated at the 5' end, and then annealed to uracil containing M13mp11E1A
- 15 single-stranded template DNA. The annealed primer/template reactions are incubated with T4 DNA polymerase, T4 DNA ligase and deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) to synthesize a complementary strand containing the E1A mutation of interest. The complementary strand synthesis reaction are then transformed into the *ung*⁺ wild type host bacterial strain, MV1190. After transformation the parental
- 20 M13mp19E1A DNA strand, which contains uracil, cannot be replicated efficiently in MV1190. Therefore the replicative form double strand DNA containing the E1A mutation of interest is enriched. M13mp19E1A phage DNA from potential E1A mutants is first screened by restriction enzyme analysis and then by DNA-sequencing, in both strands, to confirm the desired E1A-mutations.

- 25 Construction of an adenovirus comprising the E1A Δ 101/deletions is carried out by using homologous recombination in the adenovirus E1-region containing 293 cell line by the method of McGory, *et al.*, (1988) *Virology* 163, 614-617. This method requires two plasmids, one a viral plasmid containing the entire wtAd5 genome modified as in Example 1 to contain the E1B Δ 55K deletion and the MLP promoter
- 30 (pXC1-E1B55-2A), and the other a transfer plasmid containing an E1A gene with the Δ 101/07 double E1A-mutant. The transfer plasmid, pLE2 contains wtAd5 sequences from 22-1774 cloned in the tetracycline gene of pBR322 Jelsma *et al.*, (1988) *Virology* 163, 494-502. For transfer of the E1A Δ 1101 and Δ 1107 E1A-mutants from the

M13mp19 background in which they were constructed, wild E1A restriction enzyme fragments in pLE2 are replaced with cognate mutated E1A fragments from M13mp19E1 Δ 1101 and M13mp19E1A Δ 1107 to create pLE2E1A Δ 01/07. For recombination to produce adenovirus E1A Δ 01/07 the viral plasmid, pXC1-E1B55-2A and pLE2E1A Δ 01/07 are cotransfected into 293 cells by calcium phosphate mediated transfection. After 5 hours the precipitate are rinsed and the cells are overlaid with growth medium containing agarose to isolate viral plaques. At 7-10 days after the initial transfection viral plaques are isolated, plaque purified two times, and subsequently viral DNA is screened using restriction enzyme analysis and DNA sequencing. Viral stocks are purified by double cesium chloride gradients and quantitated by column chromatography as described in Huyghe, *et al.* (1995) Human Gene Therapy 6:1403-1416.

Claims

I claim:

1. A replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element.
- 5 2. The virus of claim 1 wherein the virus is an adenovirus.
3. The virus of claim 2 wherein the late regulatory element is the adenoviral major late promoter.
4. The virus of claim 3 wherein the therapeutic transgene is a tumor suppressor gene.
5. The virus of claim 4 wherein the tumor suppressor gene is p53.
- 10 6. The virus of claim 5 further comprising a deletion of E1B-55K function.
7. The virus of claim 6 further comprising a replication control sequence operably linked to an early gene.
8. The virus of claim 7 wherein the replication control sequence is a tumor specific promoter.
- 15 9. The virus of claim 7 wherein the replication control sequence is the alpha-fetoprotein.
10. The virus of claim 9 wherein the early gene is the E4 gene.
11. The virus of claim 6 further comprising a deletion of E1a 12S and 13S functions.
(01/07)
- 20 12. A pharmaceutical formulation comprising a replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element and a pharmaceutically acceptable carrier.
13. The formulation of Claim 12, further comprising a delivery enhancing agent.
14. The formulation of claim 13 wherein the delivery enhancing agent is a calpain inhibitor.
- 25 15. The formulation of claim 14 wherein the calpain inhibitor is N-acetyl-leu-leu-norcininal.
16. The formulation of Claim 13 wherein said delivery enhancing agent is a detergent.

17. A method of ablating a neoplastic cell by contacting said neoplastic cell with a replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element..
18. The method of claim 17 wherein the method is practiced *in vivo*.
- 5 19. The method of claim 18 wherein the vector is administered by intraperitoneal, intravenous or intratumoral injection.
20. The method of claim 17 wherein the method is practiced *ex vivo*.
21. The method of claim 20 wherein the method is practiced to eliminate tumor cells from stem cell products.
- 10 22. A cell transformed with a replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element..

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/26004

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N15/86 C12N15/57 C07K14/47 A61K48/00 A61P35/00 C12N5/06 C12N5/10	
According to International Patent Classification (IPC) or to both national classification and IPC	
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched	
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)	
C. DOCUMENTS CONSIDERED TO BE RELEVANT	
Category *	Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.
X	WILLS K N ET AL: "DEVELOPMENT AND CHARACTERIZATION OF RECOMBINANT ADENOVIRUSES ENCODING HUMAN P53 FOR GENE THERAPY OF CANCER" HUMAN GENE THERAPY, XX, XX, vol. 5, no. 9, 1 September 1994 (1994-09-01), pages 1079-1088, XP000579605 ISSN: 1043-0342 cited in the application
Y	abstract; figure 1 page 1081, right-hand column, paragraph 2 --- -/--
	1-6 11
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.	
<input checked="" type="checkbox"/> Patent family members are listed in annex.	
* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document relating to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "*" document member of the same patent family	
Date of the actual completion of the international search 20 April 2000	Date of mailing of the international search report 08/05/2000
Name and mailing address of the ISA European Patent Office, P.B. 5618 Patentian 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mateo Rosell, A.M.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/26004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	JELSM A ET AL., : "USE OF DELETION AND POINT MUTANTS SPANNING THE CODING REGION OF THE ADENOVIRUS 5 E1A GENE TO DEFINE A DOMAIN THAT IS ESSENTIAL FOR TRANSCRIPTIONAL ACTIVATION" VIROLOGY, vol. 163, 1988, pages 494-502, XP000905053 cited in the application the whole document ---	11
A	KANI F ET AL: "GENE THERAPY FOR ALPHA-FETOPROTEIN-PRODUCING HUMAN HEPATOMA CELLS BY ADENOVIRUS-MEDIATED TRANSFER OF THE HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE" HEPATOLOGY,US,BALTIMORE, MD, vol. 23, 1 January 1996 (1996-01-01), pages 1359-1368, XP002067680 ISSN: 0270-9139 page 1360, right-hand column, last paragraph -page 1362, left-hand column, paragraph 1 ---	2,6-9, 17-21
A	WO 95 33060 A (CARILLO SERGE ;PEICHACZYK MARC (FR); BLANCHARD JEAN MARIE (FR); RH) 7 December 1995 (1995-12-07) page 3, line 3 -page 7, line 11 page 28, line 21 -page 31, line 23 page 40, line 3 -page 41, line 21 page 43, line 26 -page 45, line 6 ---	2,6,11, 12,14, 17-20
A	WO 98 39465 A (LAMPARSKI HENRY G ;CALYDON INC (US); SCHUUR ERIC R (US); LITTLE AN) 11 September 1998 (1998-09-11) page 5, line 3-21 page 28, line 21 -page 31, line 23 page 40, line 3 -page 41, line 21 page 43, line 26 -page 45, line 6 ---	1,2,4,5, 12-14
E	WO 00 00628 A (GENVEC INC ;KOVESDI IMRE (US); BROUGH DOUGLAS E (US)) 6 January 2000 (2000-01-06) page 4, line 20 -page 5, line 9 -----	1-5

Form PCT/ISA210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/26004

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18,19
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
- ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
- ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
- ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
- ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/26004

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		AU 714209 B	23-12-1999
		AU 2620695 A	21-12-1995
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WO 9839465 A	11-09-1998	AU 6679698 A	22-09-1998
		EP 0972063 A	19-01-2000
WO 0000628 A	06-01-2000	NONE	

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